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13. ABSTRACT (Maximum 200 Words)

Angiogenesis, the formation of capillaries from pre-existing blood vessels, is essential for sustained growth of solid tumors. Numerous studies have shown that copper is required to modulate several pro-angiogenic factors. However, the specific effects of copper homeostasis on tumor angiogenesis have not been extensively studies. Our preliminary studies demonstrated that tetrathiomolybdate, a potent and novel copper chelator, blocks tumor growth and angiogenesis. We hypothesize that TM is inhibiting tumor angiogenesis by decreasing levels of VEGF, bFGF, IL-6, and IL-8 through interference with the NFkB signaling cascade. In this proposal, the molecular mechanism whereby TM regulates NFkB expression and activity will be investigated. We will establish if the NFkB transcription factor complexes, p50, p52, RelA, and RelB are regulated by TM using western blot analysis and gel shift assays. Furthermore, using a reporter gene system, we will ascertain if TM regulation of VEGF, bFGF, IL-6, and IL-8 is a direct consequence of NFkB signal inhibition. The studies as outlined will help us better understand the role of copper deficiency in tumor angiogenesis and may lead to a more specific and potent global anti-angiogenic approach to treat breast cancer.

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Introduction:

The NFκB/Rel family of transcription factors is comprised of RelA, RelB, c-Rel, p50 (nfκb1), and p52 (nfκb2) (1). In recent years, evidence linking uncontrolled NFκB activity to oncogenesis has emerged. NFκB has been shown to regulate genes important for invasion, angiogenesis, and metastasis. These include pro-angiogenic factors, such as VEGF, IL-6, and IL-8, matrix metalloproteinases, urokinase plasminogen activator (uPA), and cell adhesion molecules, such as ICAM-1 and VCAM-1 (2-6). Blocking NFκB activity in human ovarian cancer cells was shown to inhibit VEGF and IL-8 expression resulting in a decrease in tumor angiogenesis (7). Using SUM149 human inflammatory breast cancer cells, we demonstrate that p50 (nfκb1) and RelA protein levels are decreased in response to TM. Our preliminary data indicate that TM also was able to block NFκB-dependent transcription in these cells. Moreover, apoptosis was increased 2-fold in SUM149 cells following TM treatment. Taken together, our data lead us to hypothesize that TM is blocking tumor angiogenesis by decreasing levels of pro-angiogenic mediators, VEGF, bFGF, IL-6, and IL-8, and inducing apoptosis through interference with the NFκB signaling cascade. The specific aims as outlined in this proposal will help us to better understand the role of copper deficiency in tumor angiogenesis and apoptosis and may lead to a more specific approach to treat breast and other cancers.

Body:

Task 2: To determine if TM regulation of VEGF, bFGF, IL-6, and IL-8 is a direct consequence of inhibiting NFκB activity (Months 13-30):

Over this past year, we had initial difficulty in cloning in the VEGF, bFGF, IL-6, and IL-8 promoter regions into a luciferase reporter gene expression vector. We have overcome this technical problem and we will have these promoter-luciferase reportor gene expression vectors in the near future. In the meantime, we decided to continue with our proposal and made progress on Task 3 (see below).

Task 3: To determine if TM is inducing apoptosis through inhibition of NFκB activity in human breast cancers (Months 24-36):

Combination therapy of TM and doxorubicin enhances apoptosis *in vitro*. Apoptosis was determined by flow cytometry using FITC-Annexin V. As shown in Figure 1A, cells treated with 1 nM TM resulted in 3.6 ± 1.1% of apoptotic cells. A concentration-dependent induction of apoptosis was observed with doxorubicin ranging from 9.9 ± 2.9% for 0.1 μM to 16.3 ± 2.1% for 0.5 μM. Doxorubicin (0.1 μM for 72 hours) induced 75 ± 3.4% apoptosis in MDA-MB435 and 60 ± 2.1% apoptosis in MDA-MB231. This observation indicates that SUM149 cells are relatively resistant to doxorubicin-induced apoptosis when compared to other commonly used breast carcinoma cell lines. SUM149 cells treated with the combination therapy resulted in a significant, greater than additive, increase in apoptosis in comparison to either compound

administered alone; 33.8 ± 4.6% or 57.5 ± 5.2% ($p<0.01$, n=3) apoptosis was observed in cells treated with TM and 0.1 μM doxorubicin or TM and 0.5 μM doxorubicin, respectively. To determine whether the increase in apoptosis observed in the combination therapy was due to enhanced initiation of the caspase cascade, processed caspase-7 and PARP were measured (Figure 1B). Treatment with TM or doxorubicin alone did not induce significant caspase-7 or PARP cleavage. In contrast, the combination of TM and doxorubicin resulted in an increase in processed caspase-7 and PARP. These observations support the notion that the enhanced apoptosis observed with the combination therapy is due to an enhancement of caspase-mediated apoptosis.

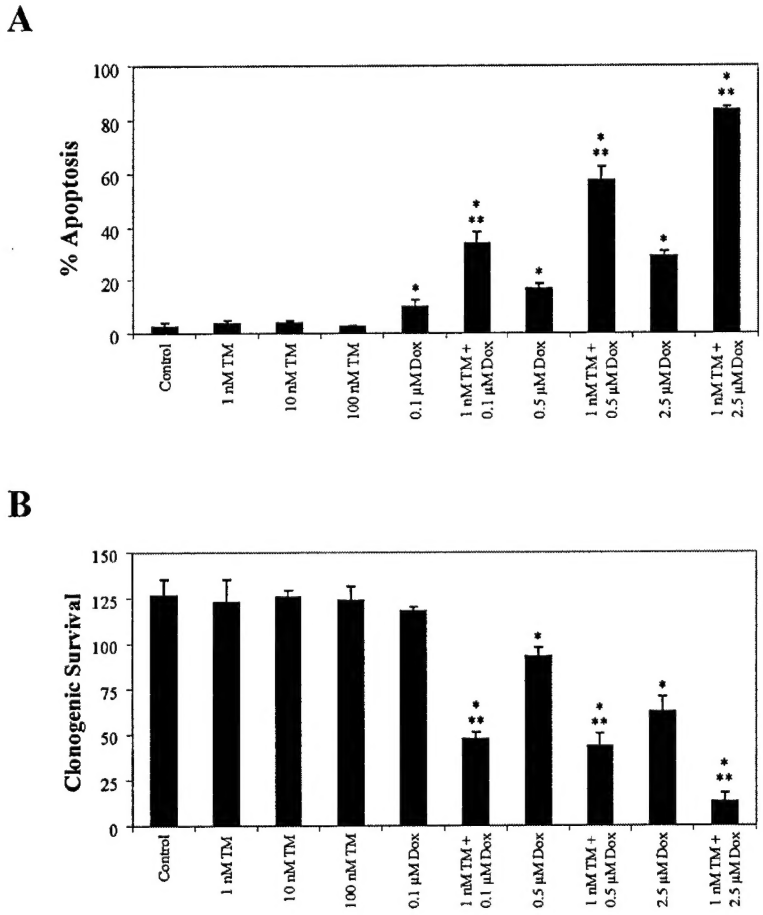

Figure 1. Combination therapy of TM and doxorubicin enhances apoptosis *in vitro*. **A**, Flow cyotometric analysis of apoptotsis. SUM149 cells (2.5×10^5) were treated with control, 1 nM TM, 0.1 μM doxorubicin, 0.5 μM doxorubicin, 1 nM TM and 0.1 μM doxorubicin, or 1 nM TM and 0.5 μM doxorubicin for 72 hours. Apoptosis was determined by the FITC-annexinV/PI assay. Data is presented as mean ± SEM. *$p<0.01$ vs. TM treatment, **$p<0.005$ vs. TM or doxorubicin treatment. **B**, Western blot analysis of processed caspase-7 and PARP. SUM149 cells (2.5×10^5) were treated with control (lane 1), 1 nM TM (lane 2), 0.1 μM doxorubicin (lane 3), or 1 nM TM and 0.1 μM doxorubicin (lane 4) for 24 hours. Total protein lysates were separated by SDS-PAGE and immunoblotted using specific antibodies to caspase-7, PARP, and β-actin. These results are representative of three independent experiments.

Combination therapy of TM and doxorubicin suppresses tumor growth and enhances apoptosis. Female athymic nude mice were transplanted with SUM149 cells (1 X 10^6) and palpable tumors were allowed to develop without treatment. At 28 days post-implantation, mice with established tumors of approximately 0.5 cm^3 were randomly assigned to four protocols and treated with control (gavaged with water daily and intravenous injection with PBS weekly), TM (1.25 mg/day loading dose for three days followed by 0.7 mg/day maintenance dose for remainder of protocol) by oral gavage, doxorubicin (5 mg/kg/week) by intravenous injection, or co-treated with TM (1.25 mg/day loading dose for three days followed by 0.7 mg/day maintenance dose for remainder of protocol) and doxorubicin (5 mg/kg/week). Although the doubling time of control SUM149 tumors appears to be around 19 days, the experiment was terminated at 28 days post-treatment due to general health concerns as the size of the mammary tumors were bulky enough to impede mice from the control group from eating and drinking. Mice treated with TM as a single agent or in combination with doxorubicin were rendered copper deficient (ceruloplasmin levels <25 ± 5% of control) after 1 week of therapy and remained so for the rest of the protocol. As shown in Figure 2, therapy with TM or doxorubicin alone significantly inhibited tumor growth in comparison to control mice ($p<0.05$, n=6). More importantly, mice treated with the combination of TM and doxorubicin resulted in complete tumor stabilization without an apparent increase in toxicity as assessed by general health, weight, and behavior ($p<0.008$ compared to control; $p<0.03$ compared to TM or doxorubicin; n=6).

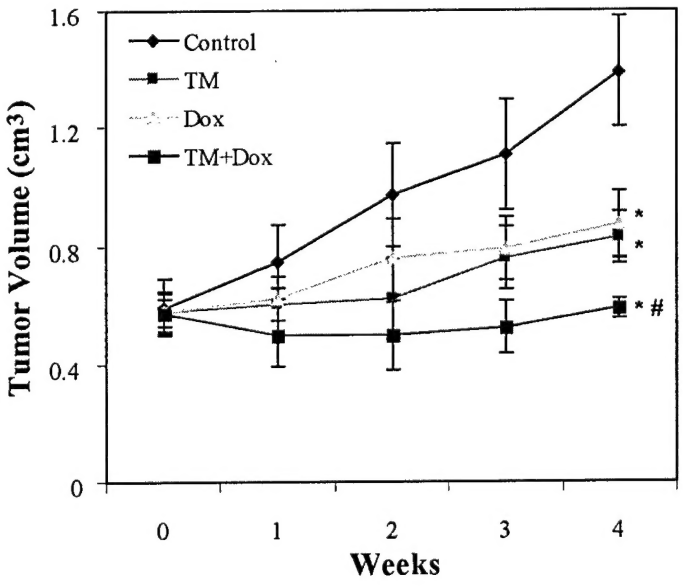

Figure 2. Combination therapy of TM and doxorubicin inhibits tumor growth in SUM149 xenografts. SUM149 cells (1 X 10^6) were orthotopically injected in the upper left mammary fat pad of 10-week old athymic female mice. At 28 days post-implantation, mice with established tumors of approximately 0.5 cm^3 were randomly assigned to four protocols; control (gavaged with water daily and intravenous injection with PBS weekly), TM (1.25 mg/day loading dose for three days followed by 0.7 mg/day maintenance dose for remainder of protocol) by oral gavage, doxorubicin (5 mg/kg/week) by intravenous injection, or co-treated with TM (1.25 mg/day loading dose for three days followed by 0.7 mg/day maintenance dose for remainder of protocol) and doxorubicin (5 mg/kg/week). Tumor volume was calculated as (length X $width^2$)/2 and presented as mean ± SEM.. *$p<0.05$ vs. control group, #$p<0.03$ vs. TM- or doxorubicin-treated group; n=6 per treatment group.

Immunohistochemical analyses of the resected tumors revealed that intratumoral apoptosis was significantly higher than control in all treatment protocols ($p<0.01$); 50% increase in the TM-treated group, 110% increase in the doxorubicin-treated group, and 220% increase in the combination-treated group. Moreover, tumor cell apoptosis was significantly higher in the combination therapy group than in either single agent group of TM or doxorubicin ($p<0.01$). As expected, tumors from the control group were highly vascularized with a mean vessel count of 33 ± 4 (mean ± SEM) per high-power field. In contrast, the smaller tumors resected from TM- (19 ± 2, $p<0.01$), doxorubicin- (23 ± 3, $p<0.05$), or TM and doxorubicin-treated (15 ± 2, $p<0.01$) mice were significantly less vascularized per high-power field. The difference in microvessel density between these three treatment groups did not reach statistical significance. Taken together, our data indicate that the combination therapy of TM and doxorubicin is more effective at suppressing tumor growth due to an increase in intratumoral apoptosis.

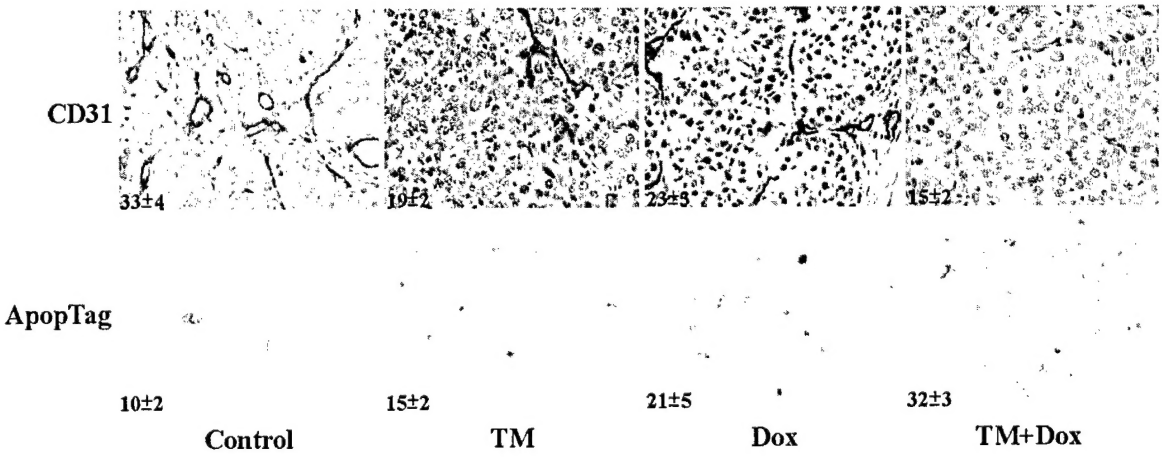

Figure 3. Immunohistochemical analyses of SUM149 xenografts. Mammary tumors from control, TM-treated, doxorubicin-treated, and TM and doxorubicin combination-treated mice were resected and processed for immunohistochemical analysis. Intratumoral blood vessels were visualized using a CD31 antibody and intratumoral cell apoptosis was visualized using the ApopTag kit. Mean vessel count per high power field (40X) and mean number of apoptotic cells per 500 total cells in a high power field (40X) was calculated and presented as mean ± SEM.

Key Research Accomplishments and Reportable Outcomes:

1. TM was found to potentiate the effect of doxorubicin on inducing tumor cell apoptosis *in vitro* and *in vivo*.

Conclusions:

We have made significant progress in the past year in determining whether TM can potentiate the efficacy of doxorubicin in promoting tumor cell apoptosis. TM was found to significantly enhance the effect of doxorubicin in inducing tumor cell apoptosis *in vitro* using annexin V and clonogenic cell survival assays and *in vivo* using an orthotopic model of breast cancer.

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